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The Regulation of Triglyceride Synthesis and Fatty Acid Synthesis in Rat Epididymal Adipose Tissue

EFFECTS OF ALTERED DIETARY AND HORMONAL CONDITIONS

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1. Epididymal adipose tissues obtained from rats that had been previously starved, starved and refed a high fat diet for 72h, starved and refed bread for 144h or fed a normal diet were incubated in the presence of insulin+glucose or insulin+glucose+acetate. 2. Measurements were made of the whole-tissue concentrations of hexose phosphates, triose phosphates, glycerol 1-phosphate, 3-phosphoglycerate, 6-phosphogluconate, adenine nucleotides, acid-soluble CoA, long-chain fatty acyl-CoA, malate and citrate after 1h of incubation. The release of lactate, pyruvate and glycerol into the incubation medium during this period was also determined. 3. The rates of metabolism of glucose in the hexose monophosphate pathway, the glycolytic pathway, the citric acid cycle and into glyceride glycerol, fatty acids and lactate+pyruvate were also determined over a 2h period in similarly treated tissues. The metabolism of acetate to CO_2 and fatty acids in the presence of glucose was also measured. 4. The activities of acetyl-CoA carboxylase, fatty acid synthetase and isocitrate dehydrogenase were determined in adipose tissues from starved, starved and fat-refed, and alloxan-diabetic animals and also in tissues from animals that had been starved and refed bread for up to 96h. Changes in these activities were compared with the ability of similar tissues to incorporate [^{14}C]glucose into fatty acids *in vitro*. 5. The activities of acetyl-CoA carboxylase and fatty acid synthetase roughly paralleled the ability of tissues to incorporate glucose into fatty acids. 6. Rates of triglyceride synthesis and fatty acid synthesis could not be correlated with tissue concentrations of long-chain fatty acyl-CoA, citrate or glycerol 1-phosphate. In some cases changes in phosphofructokinase flux rates could be correlated with changes in citrate concentration. 7. The main lesion in fatty acid synthesis in tissues from starved, starved and fat-refed, and alloxan-diabetic rats appeared to reside at the level of pyruvate utilization and to be related to the rate of endogenous lipolysis. 8. It is suggested that pyruvate utilization by the tissue may be regulated by the metabolism of fatty acids within the tissue. The significance of this in directing glucose utilization away from fatty acid synthesis and into glyceride-glycerol synthesis is discussed.

It is well known that prior manipulation of the dietary or hormonal status of rats will influence the subsequent metabolism *in vitro* of adipose tissues obtained from these animals. Previous starvation, fat feeding, or induction of alloxan-diabetes have all been shown to lead to considerable decreases in the rate of fatty acid synthesis from glucose and other precursors by adipose tissues *in vitro* (Hausberger, Milstein & Rutman, 1954; Hausberger & Milstein, 1955; Winegrad & Renold, 1958; Jeanrenaud & Renold, 1960; Leveille & Hanson, 1966). Under conditions of starvation or alloxan-diabetes, decreased rates of fatty acid synthesis by adipose tissues *in vitro* are generally accompanied by other changes in the pattern of metabolism, namely a decreased utilization of glucose by the tissue and an increased rate of lipolysis of endogenous glycerides (Randle, Garland, Hales & Newsholme, 1963; Froesch, 1965). These changes can be reversed in tissues from starved rats by refeeding and, in tissues from diabetic rats, by administration of insulin to the donor animals (Hausberger *et al.* 1954; Katz, Landua & Bartsch, 1966). Dietary or hormonally induced changes in the rate of fatty acid synthesis by adipose tissues *in vitro*, despite the importance of these tissues as a major site of lipogenesis, appear to have been considered to a far lesser extent than those in liver where such effects

are well documented (Korchak & Masoro, 1962; Wieland & Eger-Neufeldt, 1963; Bortz, Abraham & Chaikoff, 1963).

Several factors could influence the metabolism of hexose to fatty acids by adipose tissues *in vitro*. As discussed by Winegrad (1965), it is possible that the capacity for uptake of glucose by tissues *in vitro* may be influenced by their previous dietary and hormonal status, with consequent changes occurring in the pathways of hexose utilization. Alterations in dietary and hormonal status are known to produce changes in the activities of several enzymes of the lipogenic pathways which usually parallel changes in the lipogenic capacity *in vitro*. ATP–citrate lyase, 'malic enzyme', hexokinase and the hexose monophosphate pathway dehydrogenases all exhibit apparently adaptive behaviour (Young, Shrago & Lardy, 1964; Moore, Chandler & Tettenhorst, 1964; Wise & Ball, 1964; Kornacker & Ball, 1965; Hollifield & Parson, 1965; Anderson & Hollifield, 1966; McLean, Brown, Greenslade & Brew, 1966; Katzen, 1966; Borrebaek, 1966) similar to that found in liver. It does not appear to have been established, however, whether the activities of acetyl-CoA carboxylase and the fatty acid synthetase complex of adipose tissue show adaptive responses to changes in dietary and hormonal status as is the case for the liver enzymes (Gibson & Hubbard, 1960; Hubbard, Allman, McLain & Gibson, 1961; Numa, Matsuhashi & Lynen, 1961; Wieland, Neufeldt, Numa & Lynen, 1962; Bortz *et al.* 1963; Allman, Hubbard & Gibson, 1965). Adipose tissues from rats of varied dietary or hormonal status may also have altered intracellular metabolite concentrations which may be critical in determining rates of fatty acid and triglyceride synthesis. In particular, citrate has been shown to be both an activator of adipose tissue acetyl-CoA carboxylase (Martin & Vagelos, 1962; Vagelos, Alberts & Martin, 1963) and an inhibitor of adipose tissue phosphofructokinase (Denton & Randle, 1966) in purified systems. The concentrations of long-chain fatty acyl-CoA derivatives, free fatty acids and glycerol 1-phosphate have also been proposed to be of importance in the regulation of lipogenesis (Bortz & Lynen, 1963; Numa, Bortz & Lynen, 1964; Korchak & Masoro, 1964; Kipnis & Kalkoff, 1965; Howard & Lowenstein, 1964, 1965).

It was the intention of the present study to attempt to correlate changes in fatty acid synthesis from glucose and from acetate over a range of physiological states with changes in the intracellular concentrations of possible regulatory metabolites. Studies of metabolite concentrations may also permit the location of possible regulatory enzymes (Rolleston & Newsholme, 1967; Newsholme & Gevers, 1967). Measurements have also been made to determine whether acetyl-CoA carboxylase and the fatty acid synthetase complex show adaptive changes in their activities with the intention of attempting to correlate changes in the contents of these, and other known adaptive enzymes, with altered rates of lipogenesis *in vitro* and with altered metabolite concentrations *in vitro* in adipose tissues obtained from animals of varied hormonal and dietary status.

Experiments involving measurements of the tissue contents of metabolites in normal adipose tissues maintained *in vitro* in a range of steady states through the inclusion of various hormones or metabolites in the incubation medium, and correlation of these contents with altered rates of triglyceride synthesis from glucose have been undertaken previously in attempts to elucidate points of control in the metabolism of glucose to triglyceride (Denton, Yorke & Randle, 1966; Denton & Halperin, 1968; Halperin & Denton, 1969; Saggerson & Greenbaum, 1970). In the present study use is made of prior dietary or hormonal manipulations, rather than addition of substances to the incubation medium, to vary the steady states studied *in vitro*.

MATERIALS AND METHODS

Chemicals. Reagents and enzymes were obtained or prepared as described by Saggerson & Greenbaum (1970). In addition, trisodium DL-isocitrate was obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. Acetyl-CoA was synthesized by the method of Simon & Shemin (1953) and standardized by the method of Stadtman (1957) by using phosphotransacetylase (EC 2.3.1.8). Malonyl-CoA was prepared by the reaction of CoASH and *S*-malonyl-*N*-octanoyl thioethanolamine as described by Lynen (1962). *S*-Malonyl-*N*-octanoyl thioethanolamine (m.p. 69–70°C) was prepared by Dr P. B. Bunyan. The malonyl-CoA was standardized by the hydroxamic acid procedure of Stadtman (1957). Acetyl-CoA and malonyl-CoA were stored in solution at pH 5.5 and −20°C.

Animals. Male albino rats of an inborn strain were used in all experiments. The animals were maintained on cube diet 41B (Bruce & Parkes, 1949) and were supplied with water *ad libitum*.

Treatment of animals. Animals used for measurements of enzyme activities weighed between 145 and 155g at the commencement of treatment. Animals weighing between 150 and 180g were selected for measurement of tissue metabolite contents and for measurement of carbon fluxes. The methods of starvation, starvation and refeeding with high-fat or bread diets, the induction of alloxan-diabetes and the treatment of alloxan-diabetic animals with insulin were as described by Saggerson & Greenbaum (1969). Animals designated 'controls' or 'normal' were maintained on cube diet 41B throughout.

Determination of tissue metabolite contents in vitro. The incubation of fat pads, the extraction and assay of metabolic intermediates in them, the analysis of incubation media and the determination of tissue [^{14}C]sorbitol, [^{3}H]-

water and glucose spaces were all performed as described by Saggerson & Greenbaum (1970).

Extraction of acetyl-CoA carboxylase, fatty acid synthetase and isocitrate dehydrogenase (NADP). Animals were killed by cervical dislocation. The epididymal fat pads were immediately excised, trimmed free of blood vessels as far as possible, rinsed and homogenized in ice-cold 0.25M-sucrose containing 0.5mM-dithiothreitol and 2mM-EDTA (5ml/g of tissue). In all cases the homogenate was made from the entire epididymal adipose tissue obtained from one animal, except for a small piece of tissue (20–30mg) which was set aside for determination of tissue nitrogen. Homogenization was in a glass Potter–Elvehjem homogenizer cooled in ice, fitted with a motor-driven Teflon pestle with a clearance of 0.5 mm. The homogenate was centrifuged at 105000*g* for 30min at 2–4°C in a no. 50 rotor of a Spinco model L preparative ultracentrifuge. The resulting aqueous fraction was decanted off and dialysed for 2h at 4°C in 8/32in Visking seamless dialysis tubing that had been previously boiled in 10mM-EDTA and well rinsed with water. The dialysis medium was 100vol. of 50mM-triethanolamine buffer, pH7.4, containing 2mM-EDTA and 0.25mM-dithiothreitol. The extracts were found to increase in volume by approx. 25% during dialysis. All dialysed high-speed supernatants were kept in ice until used, and all assays were performed within 6h, during which time no loss of enzyme activity was observed. Control experiments, involving the re-extraction of the fat plug produced by the centrifugation, showed that 7% of the total extractable fatty acid synthetase activity and only about 1% of the acetyl-CoA carboxylase and isocitrate dehydrogenase (NADP) activities were retained in the fat plug. Other control experiments indicated that the 0.25M-sucrose medium was slightly better than a 0.15M-KCl-based medium (Saggerson & Greenbaum, 1969) in extraction of acetyl-CoA carboxylase and fatty acid synthetase from the tissues. No correction was made for the contribution of the very small amount of adipose tissue water (Crofford & Renold, 1965) to the total volumes of the homogenates.

Assay of fatty acid synthetase and isocitrate dehydrogenase (NADP). The enzyme measurements were made by recording rates of change in extinction at 340nm with a Unicam SP.800 recording spectrophotometer at 25°C. All reactions were carried out in 3ml volumes in cuvettes of 1cm light-path. In all cases the rates of reaction were constant over the measured period (3–5min). Preliminary experiments indicated that in all cases the rates of reaction were proportional to the amounts of dialysed high-speed supernatant used, and that the concentrations of substrates and pH of buffers used gave maximal activities. Reactions were started by the addition of the necessary amount of dialysed high-speed supernatant. Simultaneous blanks were carried out by addition of tissue extract to an identical cuvette from which a substrate was omitted.

The methods used were basically those of Gibson & Hubbard (1960) and Ochoa (1955). The components of the assay mixtures were as follows.

Fatty acid synthetase. 100μmol of potassium phosphate buffer, pH6.5, 0.5μmol of $NADP^+$, 75nmol of malonyl-CoA, 50nmol of acetyl-CoA and 0.1–0.4ml of dialysed high-speed supernatant.

Isocitrate dehydrogenase (NADP) (EC 1.1.1.42). 100μmol of tris–HCl buffer, pH7.4, 10μmol of $MgCl_2$, 1μmol of $NADP^+$ and 0.1–0.2ml of dialysed high-speed supernatant.

Assay of acetyl-CoA carboxylase (EC. 6.4.1.2). The method used was basically that of Martin & Vagelos (1962) and measured the incorporation of [^{14}C]bicarbonate into acid-stable material. Samples (0.1 or 0.2ml) of dialysed high-speed supernatants were placed in 10ml glass centrifuge tubes at 37°C. After 2min the following was added to give a final volume in each tube of 1.0ml: 40μmol of triethanolamine buffer, pH7.4, 80μmol of potassium citrate, pH7.4, 20μmol of $MgCl_2$, 8μmol of $MnCl_2$, 30μmol of $KH^{14}CO_3$ (2μCi), 10μmol of ATP, 1μmol of dithiothreitol and 0.2μmol of acetyl-CoA. The incubation was carried out for 10min at 37°C and was then stopped by the addition of 0.2ml of acetic acid–HCl (10:1, v/v). The tubes were placed in ice, and 0.1ml samples of the acidified solutions were spread on to circles of Whatman no. 1 filter paper (3cm diam.), suspended in a hot-air blast, and evaporated to dryness. The paper circles were immersed in 15ml of a scintillator consisting of 4g of 2,5-bis-(5-*tert.*-butylbenzoxazol-2-yl)thiophen/litre of toluene, and counted for radioactivity in a Nuclear–Chicago liquid-scintillation counter. Observed count rates were corrected to d.p.m. by the 'channels ratio' method of Baillie (1960). Corrections were also made for the background count rate. It was observed that the area of the paper circle, at least up to 8cm^2, did not affect the counting, nor did the orientation of the paper with respect to the phototubes of the counter. However, only approx. 80% of the d.p.m. of a standard ^{14}C-containing sample could be detected when counted on these papers. A correction factor was therefore used in the calculation of results. Control experiments showed that there was no retention of ^{14}C-containing material on the papers in incubations lacking the addition of dialysed high-speed supernatant. Acid-stable radioactivity was taken to represent malonyl-CoA (Martin & Vagelos, 1962; Levy, 1963).

The concentrations of substrates and cofactors used in the assay were those that gave maximum, or near maximum, incorporation of [^{14}C]bicarbonate. Although it was essential that citrate be present in this assay, little was gained, in terms of extra activation, from preincubating the enzyme with citrate, unlike the systems of Martin & Vagelos (1962) or Levy (1963). This lack of an appreciable time-dependence of citrate activation is presumably due to the far higher citrate concentrations used in the present study. Fang & Lowenstein (1967) have demonstrated that the extent of citrate activation of acetyl-CoA carboxylase in rat liver extracts is dependent on the citrate concentration and on the time of activation in an interdependent manner. Control experiments indicated that the incorporation of [^{14}C]bicarbonate into acid-stable material was proportional to the amount of dialysed high-speed supernatant used, and that this incorporation was linear with time.

Determination of ^{14}C flux rates in incubated tissue segments. Incubation of tissue segments with various substrates, extraction of tissues, measurements of the yields of ^{14}C in various products and calculation of flux rates were performed as described by Saggerson & Greenbaum (1970).

Determination of tissue nitrogen. This was performed as described by Saggerson & Greenbaum (1969), either on

20–30 mg samples of powdered frozen fat pads in experiments to determine tissue metabolite contents, or in 20–30 mg samples of fresh tissue in experiments to determine enzyme activities or carbon fluxes.

RESULTS

Measurements of metabolic intermediates in incubation media and in adipose tissues, from rats of varied dietary status, incubated in the presence and absence of acetate. Four experiments were performed in which whole-tissue concentrations of metabolites were measured. Basic data concerning the animals used in these experiments are shown in Table 1. Table 1 also records data concerning the release of lactate, pyruvate and glycerol by whole, pooled tissues incubated for 1 h. Similar data on lactate, pyruvate and glycerol release by single fat pad segments incubated for 2 h are also shown in Tables 7 and 9. The results of Tables 1 and 7 are in agreement in showing that glycerol release is increased in tissues from starved rats with respect to those from normal rats, and that refeeding with a high-fat diet does not decrease the release of this metabolite, whereas refeeding with bread supresses the increased glycerol release incurred on starvation within 24 h (Table 7). In all three experimental treatments shown in Table 1, the release of lactate + pyruvate by the incubated tissues was increased above normal; this is also shown in Tables 7 and 9. Incubation in the presence of sodium acetate was found to decrease this elevated lactate + pyruvate efflux in the case of tissues from starved and starved, fat-refed rats (Tables 1 and 9), but to increase it in the case of tissues from starved, bread-refed rats. Similarly, though to a less significant extent, incubation of tissues from starved, or starved, fat-refed rats with acetate was found to decrease the release of glycerol, whereas the release of glycerol was increased when tissues from starved, bread-refed rats were incubated with acetate (Table 1). The effects of sodium acetate on the metabolism of tissues from alloxan-diabetic rats was not studied; however, it may be seen in Table 7 that, as in the case of tissues from starved rats, the release of lactate + pyruvate and glycerol is considerably increased in tissues from alloxan-diabetic rats. Treatment of alloxan-diabetic animals with insulin for 24 h before incubation of the tissues led to a normalization of glycerol release and a lowering of the lactate + pyruvate production to near-normal levels.

Whole-tissue concentrations of various phosphorylated sugars in incubated tissues of varied dietary status are shown in Table 2. In general, the concentrations of these intermediates per mg of tissue N did not vary greatly between the experimental groups. However, tissues from starved rats

Table 1. *Effects of alteration in dietary status and addition of sodium acetate on release of lactate, pyruvate and glycerol by incubated epididymal adipose tissues*

Details of incubation procedures are as described in the Materials and Methods section. The results are given as mean values ± S.E.M. The group of tissues from animals fed the 'normal' diet were taken as controls for those groups of tissues obtained from animals of different dietary status which were incubated with insulin alone. Groups of tissues of varied dietary status incubated with insulin and sodium acetate were compared with tissues of the same dietary status incubated with insulin alone, as controls. * $P<0.05$; † $P<0.01$; ‡ $P<0.001$ (versus the appropriate control).

Expt. no.	Dietary status of animals	Additions to incubation medium	Metabolite released (μmol/h per mg of tissue N)			Mean body wt. of animals (g)	Total no. of animals	No. of groups of pooled tissues	Mean wt. of tissues from each animal (mg)
			Pyruvate	Lactate	Glycerol				
1	Normal	Insulin	0.19 ± 0.01	1.40 ± 0.20	0.19 ± 0.04	117 ± 2	28	5	1045
2	Starved for 72 h	Insulin	0.39 ± 0.03‡	3.84 ± 0.28‡	0.62 ± 0.03‡	130 ± 1	48	6	596
		Insulin + acetate	0.23 ± 0.01‡	2.65 ± 0.24†	0.55 ± 0.03				
3	Starved for 72 h and refed high-fat diet for 72 h	Insulin	0.15 ± 0.02	2.04 ± 0.21	0.49 ± 0.04‡	146 ± 1	40	5	623
		Insulin + acetate	0.15 ± 0.02	1.49 ± 0.21	0.42 ± 0.05				
4	Starved for 72 h and refed bread for 144 h	Insulin	0.36 ± 0.02‡	2.31 ± 0.17†	0.23 ± 0.02	150 ± 1	48	6	916
		Insulin + acetate	0.80 ± 0.05‡	3.93 ± 0.43†	0.28 ± 0.01*				

Table 2. *Effects of alteration of dietary status and addition of sodium acetate on the whole-tissue concentrations of phosphorylated sugars in incubated epididymal adipose tissues*

Details of incubation procedures are described in the Materials and Methods section. The results are given as mean values±S.E.M. The group of tissues from animals fed the 'normal' diet was taken as control for those groups of tissues obtained from animals of different dietary status which were incubated with insulin alone. Groups of tissues of varied dietary status incubated with insulin and sodium acetate were compared against tissues of the same dietary status incubated with insulin alone, as controls. The following abbreviations are used in this and subsequent tables: 6PG, 6-phosphogluconate; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; DHAP, dihydroxyacetone phosphate; G1P, glycerol 1-phosphate; 3PG, 3-phosphoglycerate; GAP, glyceraldehyde 3-phosphate. *$P<0.01$; †$P<0.001$ (versus the appropriate control).

Expt. no.	Dietary status of animals	Additions to incubation medium	Metabolite concentration (nmol/mg of tissue N)							
			6PG	G6P	F6P	FDP	DHAP	G1P	3PG	GAP
1	Normal	Insulin	3.75±0.25	6.01±0.59	2.10±0.28	1.30±0.17	2.10±0.20	28.7±3.0	4.10±0.70	—
2	Starved for 72h	Insulin	1.30±0.32†	4.50±0.61	1.76±0.18	1.34±0.22	2.50±0.21	32.7±1.2	3.00±0.26	—
		Insulin + acetate	1.45±0.24	4.85±0.52	2.16±0.25	1.09±0.18	2.05±0.59	32.9±7.0	2.53±0.21	—
3	Starved for 72h and refed with high-fat diet for 72h	Insulin	4.80±0.54	7.19±0.61	2.67±0.14	1.73±0.23	4.06±0.41*	72.7±7.5†	3.58±0.40	—
		Insulin + acetate	5.31±0.47	6.94±0.50	2.44±0.17	1.28±0.27	3.71±0.74	56.5±8.6	3.16±0.35	—
4	Starved for 72h and refed with bread for 144h	Insulin	10.31±0.73†	8.25±0.85	2.66±0.22	1.24±0.18	3.47±0.39*	35.1±6.3	4.40±0.16	0.78±0.19
		Insulin + acetate	14.80±1.90	7.73±0.97	2.61±0.12	1.17±0.16	2.69±0.34	38.2±4.0	4.19±0.41	0.58±0.14

Table 3. *Effects of alteration of dietary status and addition of sodium acetate on the whole tissue concentrations of adenine nucleotides in incubated epididymal adipose tissues*

Details of incubation procedures are described in the Materials and Methods section. The results are given as mean values±S.E.M. The group of tissues from animals fed the 'normal' diet were taken as controls for those groups of tissues obtained from animals of different dietary status which were incubated with insulin alone. Groups of tissues of varied dietary status incubated with insulin and sodium acetate were compared with tissues of the same dietary status incubated with insulin alone, as controls. *$P<0.05$; †$P<0.01$ (versus the appropriate control).

Expt. no.	Dietary status of animals	Additions to incubation medium	Metabolite concentration (nmol/mg of tissue N)				Ratio		
			ATP	ADP	AMP	(ATP+ADP+AMP)	ATP	: ADP	: AMP
1	Normal	Insulin	89.8±9.0	21.7±2.4	22.2±3.6	133.7±13.7	10	: 2.4	: 2.5
2	Starved for 72h	Insulin	66.2±4.7*	17.1±3.0	11.7±5.1	95.0±7.2*	10	: 2.6	: 1.9
		Insulin + acetate	61.9±3.6	20.9±2.2	14.1±4.4	96.8±5.4	10	: 3.4	: 2.3
3	Starved for 72h and refed with high-fat diet for 72h	Insulin	84.3±5.6	18.2±0.9	11.4±2.2*	113.9±6.7	10	: 2.2	: 1.4
		Insulin + acetate	78.2±4.0	18.8±1.5	12.2±3.0	109.2±4.1	10	: 2.1	: 1.6
4	Starved for 72h and refed with bread for 144h	Insulin	79.7±6.2	16.8±1.3	4.3±1.7†	100.8±6.1	10	: 2.1	: 0.5
		Insulin + acetate	70.8±5.0	16.7±2.7	4.3±2.2	92.9±4.0	10	: 2.4	: 0.6

Table 4. *Effects of alteration of dietary status and addition of sodium acetate on the whole-tissue concentrations of long-chain fatty acyl-CoA, CoASH, malate and citrate in incubated epididymal adipose tissues*

Details of incubation procedures are described in the Materials and Methods section. The results are given as mean values ± S.E.M. The group of tissues from animals fed the 'normal' diet was taken as control for those groups of tissues obtained from animals of different dietary status which were incubated with insulin alone. Groups of tissues of various dietary status incubated with insulin and sodium acetate were compared with tissues of the same dietary status incubated with insulin alone, as controls. * $P<0.05$; † $P<0.01$; ‡ $P<0.001$ (versus the appropriate control).

Expt. no.	Dietary status of animals	Additions to incubation medium	Metabolite concentration (nmol/mg of tissue N)				
			Long-chain fatty acyl-CoA	Acid-soluble CoASH	Total CoA	Malate	Citrate
1	Normal	Insulin	0.82 ± 0.15	2.20 ± 0.16	3.03 ± 0.26	8.82 ± 1.64	9.64 ± 0.50
2	Starved for 72 h	Insulin	0.87 ± 0.08	0.95 ± 0.13‡	1.82 ± 0.15†	6.23 ± 0.70	22.5 ± 3.0†
		Insulin + acetate	0.67 ± 0.05	0.91 ± 0.09	1.58 ± 0.07	8.00 ± 0.79	26.1 ± 2.7
3	Starved for 72 h and refed high-fat diet for 72 h	Insulin	0.65 ± 0.10	2.22 ± 0.19	2.86 ± 0.28	6.25 ± 0.46	31.4 ± 5.8†
		Insulin + acetate	0.81 ± 0.04	2.30 ± 0.13	3.11 ± 0.10	7.06 ± 0.52	40.0 ± 4.8
4	Starved for 72 h and refed bread for 144 h	Insulin	1.04 ± 0.18	3.58 ± 0.28†	4.62 ± 0.40†	21.3 ± 3.8*	13.2 ± 1.9
		Insulin + acetate	1.22 ± 0.23	3.39 ± 0.19	4.61 ± 0.42	18.6 ± 1.5	17.0 ± 1.8

exhibited significantly decreased concentrations of 6-phosphogluconate (in fact this represents the sum of 6-phosphogluconate + 6-phosphogluconolactone) and tissues from starved, bread-refed rats exhibited greatly elevated concentrations of this metabolite. Significant alterations from normal in the concentrations of glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate and 6-phosphogluconate were not observed for any of the experimental groups, although the concentrations of glucose 6-phosphate and fructose 6-phosphate appeared to be consistently greater than normal in tissues from starved, fat-refed and starved, bread-refed rats, and to be consistently below normal in tissues from starved rats. The concentrations of dihydroxyacetone phosphate and glycerol 1-phosphate were quite significantly elevated in tissues from starved, fat-refed rats, whereas the concentration of dihydroxyacetone phosphate was elevated in tissues from starved, bread-refed rats. Incubation with sodium acetate produced no statistically significant changes in the concentrations of any of the phosphorylated sugars, although 6-phosphogluconate concentrations were slightly increased in the presence of acetate in all three experimental groups and the concentrations of glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate, dihydroxyacetone phosphate and 3-phosphoglycerate, were generally decreased by incubation with acetate.

Table 3 shows that all the experimental groups had lower AMP concentrations than normal, though only significantly so in the starved, refed groups. Incubation with acetate produced no noticeable effect on the adenine nucleotide pattern.

Table 4 shows that the three experimental groups of tissues had concentrations of long-chain fatty acyl-CoA similar to normal tissues, but that the acid-soluble CoA concentrations were decreased in tissues from starved rats, and were greater than normal in tissues from starved, bread-refed rats. The values of total CoA were thus decreased and elevated in these tissues respectively. Tissues from starved and starved, fat-refed rats had normal malate concentrations, but the concentration of this metabolite was elevated in tissues from starved, bread-refed rats. On the other hand, citrate concentrations were elevated in tissues from starved rats and in those from starved, fat-refed animals, but were not significantly altered from normal in tissues from starved, bread-refed rats. Incubation with acetate produced no significant changes in the concentrations of the metabolites in Table 4 in any of the experimental groups, although the concentrations of citrate were consistently raised in the presence of acetate.

Intracellular water spaces and glucose-[^{14}C]sorbitol spaces in incubated adipose tissues from rats of varied

Table 5. *Effects of alteration of dietary status on measurements of intracellular water spaces and glucose–[^{14}C]sorbitol spaces in incubated epididymal adipose tissues*

Incubation procedures and calculation of tissue spaces were as described in the Materials and Methods section. All results are mean values ± S.E.M. '*P*' values were only calculated for results expressed per mg of tissue N. $*P<0.05$; $\dagger P<0.01$; $\ddagger P<0.001$ (versus the results for tissues taken from normally fed animals)

Dietary status of animals	Mean body wt. of animals (g)	Mean wt. of pair of fat pads (mg)	No. of determinations	Additions to incubation medium	[^{3}H]Water–[^{14}C]sorbitol space (μl/g wet wt.)	[^{3}H]Water–[^{14}C]sorbitol space (μl/mg of tissue N)	Glucose–[^{14}C]sorbitol space (μl/mg of tissue N)
Normal	163 ± 3	1232 ± 44	10	Insulin + sorbitol	33.1 ± 2.3	13.5 ± 1.0	−10.9 ± 1.5
Starved for 72 h	111 ± 3	491 ± 54	8	Insulin + sorbitol	42.7 ± 5.1	12.6 ± 1.8	−5.6 ± 1.6*
Starved for 72 h and refed high-fat diet for 72 h	152 ± 2	1126 ± 126	8	Insulin + sorbitol	24.2 ± 3.9	14.7 ± 2.7	−29.9 ± 4.6†
Starved for 72 h and refed bread for 144 h	142 ± 2	1018 ± 44	8	Insulin + sorbitol	43.1 ± 3.3	15.4 ± 1.2	−23.2 ± 1.9‡

dietary status. The results of Table 5 indicate that, expressed per mg of tissue N, the total intracellular water space of the three groups of experimentally treated tissues were not significantly different from normal. Significant variations between the experimental groups in the value of glucose–[^{14}C]sorbitol space were, however, found.

Enzyme mass-action ratios in incubated adipose tissues from rats of varied dietary status. Mass-action ratios (Γ) for the phosphofructokinase, adenylate kinase and phosphoglucose isomerase reactions are presented in Table 6. These ratios in the experimentally treated tissues in the presence and absence of acetate were very similar to those in normal tissues. Values for the combined mass-action ratio (aldolase × triosephosphate isomerase) are also presented and these are also similar to those found in normal tissues (Saggerson & Greenbaum, 1970). With tissues from starved, bread-refed rats it was found possible to assay glyceraldehyde 3-phosphate (Table 2), therefore allowing an estimate of the separate Γ values for aldolase and triosephosphate isomerase to be made for these tissues. The mass-action ratio for triosephosphate isomerase in the tissues from starved, bread-refed rats was found to be 6.21 ± 2.13 for tissues incubated with insulin alone, and 5.55 ± 1.13 for the same tissues incubated with insulin + acetate. These values may be compared with values for the apparent equilibrium constant of this reaction which range from 20 to 28 (Burton, 1957; Lowry & Passonneau, 1964). Mass-action ratios for the aldolase reaction in tissues from starved, bread-refed rats were 1.4×10^{-4} M in the presence of insulin alone and 8.7×10^{-5} M in the presence of insulin + acetate. These values may be compared with published values of the apparent equilibrium constant of the reaction which range from 6.8×10^{-5} M to 1.33×10^{-4} M (Herbert, Gordon, Sabrahmanyan & Green, 1940; Meyerhof & Junowicz-Kocholaty, 1943; Krebs & Kornberg, 1957; Hess, 1963; Lowry & Passonneau, 1964).

[Lactate]/[pyruvate] ratios in incubation media, and calculated extramitochondrial free [NAD^+]/[NADH] ratios in epididymal adipose tissues from rats of varied dietary status, incubated in the presence and absence of acetate. When incubated in the presence of insulin alone, for either 1 h as whole tissues (Table 6), or for 2 h as small segments of tissue (Table 7), tissues from starved rats demonstrated a slightly increased lactate/pyruvate ratio compared with normal tissues. Refeeding starved animals with bread led first to a significant increasing of the [lactate]/[pyruvate] ratio compared with normal tissues after 24 h of refeeding (Table 7). However, after 144 h of refeeding with bread the [lactate]/[pyruvate] ratio was generally lower than in normal tissues (Tables 6, 7 and 9). Tissues from starved,

Table 6. *Enzyme mass-action ratios derived from whole-tissue metabolite concentrations measured in incubated epididymal adipose tissues from rats of varied dietary status*

Metabolite concentrations were derived from Tables 1, 2, 3, and 4. The calculation of Γ for (aldolase × triose phosphate isomerase) required the use of appropriate intracellular water-space values from Table 5. All results are given as mean values ± S.E.M. except for the $[DHAP]^2/[FDP]$ values. The group of tissues from animals fed the normal diet was taken as control for those groups of tissues obtained from animals of different dietary status which were incubated with insulin alone. Groups of tissues from rats of varied dietary status incubated with insulin + acetate were compared with tissues from rats of the same dietary status incubated with insulin alone, as controls. Extramitochondrial free $[NAD^+]/[NADH]$ ratios were calculated, assuming an intracellular pH of 7.0, as described by Hohorst, Kreutz & Reim (1961), assuming apparent equilibrium constants for lactate dehydrogenase and glycerol 1-phosphate dehydrogenase of 1.11×10^{-4} and 8.9×10^{-5} respectively (37°C, ionic strength 0.25) (Williamson, Lund & Krebs, 1967; Hohorst *et al.* 1961). *$P<0.05$; †$P<0.01$ (versus the appropriate control).

			Enzyme mass-action ratios (Γ)				Extramitochondrial free $[NAD^+]/[NADH]$ ratios calculated from	
Expt. no.	Dietary status of animals	Additions to incubation medium	Phosphofructokinase: $\frac{[FDP][ADP]}{[F6P][ATP]}$	Adenylate kinase: $\frac{[ATP][AMP]}{[ADP^2]}$	Phosphoglucose isomerase: $\frac{[F6P]}{[G6P]}$	Aldolase × triose phosphate isomerase: $\frac{[DHAP]^2}{[FDP]}$	$\frac{[Lactate]}{[pyruvate]}$	$\frac{[G1P]}{[DHAP]}$
1	Normal	Insulin	0.16 ± 0.03	4.66 ± 1.19	0.35 ± 0.04	2.3×10^{-4} M	1212 ± 197	775 ± 178
2	Starved for 72 h	Insulin	0.21 ± 0.06	2.25 ± 0.55	0.41 ± 0.05	3.7×10^{-4} M	895 ± 84	780 ± 98
		Insulin + acetate	0.14 ± 0.02	2.58 ± 0.46	0.44 ± 0.02	3.1×10^{-4} M	793 ± 65	564 ± 200
3	Starved for 72 h and refed high-fat diet for 72 h	Insulin	0.15 ± 0.03	2.87 ± 0.58	0.38 ± 0.02	6.5×10^{-4} M	671 ± 36*	610 ± 72
		Insulin + acetate	0.15 ± 0.06	2.65 ± 0.71	0.35 ± 0.02	7.3×10^{-4} M	869 ± 53*	647 ± 149
4	Starved for 72 h refed bread for 144 h	Insulin	0.11 ± 0.03	1.67 ± 0.52*	0.33 ± 0.01	6.3×10^{-4} M	1392 ± 75	1130 ± 109
		Insulin + acetate	0.14 ± 0.06	1.28 ± 0.58	0.32 ± 0.02	4.0×10^{-4} M	1838 ± 102†	758 ± 87*

Table 7. *Effects of alteration of dietary and insulin status on the incorporation of [1,6-^{14}C]glucose into fatty acids, lactate and pyruvate, and on the release of glycerol by incubated segments of epididymal adipose tissue*

Treatment of animals, incubation procedures and determination of [^{14}C] fatty acids, lactate, pyruvate and glycerol were as described in the Materials and Methods section. All incubations were performed in the presence of insulin. The results are mean values ± S.E.M. *$P<0.05$; †$P<0.01$; ‡$P<0.001$ (versus the normally fed control).

Dietary status of animals	No. of animals	Mean body wt. of animals (g)	Mean total weight of fat pads (mg)	Incorporation of [1,6-^{14}C]-glucose into fatty acids (μmol/h per mg of tissue N)	Total recovery of lipid fractions %	Release into incubation medium of: Lactate + pyruvate (μmol/h per mg of tissue N)	Release into incubation medium of: Glycerol (μmol/h per mg of tissue N)	Lactate / pyruvate ratio	Lactate + pyruvate / fatty acid ratio
Normal	28	178 ± 3	1262 ± 55	2.63 ± 0.25	96	1.63 ± 0.10	0.18 ± 0.01	5.54 ± 0.34	0.62
Starved for 72 h	7	121 ± 3	594 ± 91	0.52 ± 0.18‡	93	3.07 ± 0.68*	0.57 ± 0.10‡	6.33 ± 0.52	5.90
Starved for 72 h and refed bread for 24 h	7	138 ± 2	568 ± 81	0.89 ± 0.19‡	95	1.22 ± 0.12*	0.12 ± 0.01‡	7.51 ± 0.38‡	1.37
Starved for 72 h and refed bread for 48 h	7	134 ± 2	601 ± 36	2.64 ± 0.41	98	1.88 ± 0.27	0.13 ± 0.01†	5.53 ± 0.83	0.71
Starved for 72 h and refed bread for 72 h	7	137 ± 2	612 ± 62	2.15 ± 0.19	98	1.76 ± 0.20	0.13 ± 0.01†	6.08 ± 0.42	0.82
Starved for 72 h and refed bread for 96 h	6	139 ± 3	791 ± 78	3.06 ± 0.26	100	2.10 ± 0.18*	0.16 ± 0.01	5.02 ± 0.27	0.69
Starved for 72 h and refed bread for 144 h	7	154 ± 2	1178 ± 94	4.66 ± 0.38‡	96	2.28 ± 0.17†	0.16 ± 0.01	4.05 ± 0.23†	0.49
Starved for 72 h and refed high-fat diet for 72 h	7	159 ± 2	1210 ± 118	0.74 ± 0.06‡	91	2.64 ± 0.44*	0.46 ± 0.08†	12.52 ± 0.76‡	3.56
Fed high-fat diet for 72 h	7	156 ± 4	1464 ± 114	1.59 ± 0.23†	101	1.66 ± 0.16	0.25 ± 0.02	10.19 ± 0.63‡	1.04
Alloxan-diabetic	7	135 ± 4	926 ± 84	0.41 ± 0.09‡	79	4.15 ± 0.86†	0.83 ± 0.07‡	6.87 ± 0.63	10.1
Alloxan-diabetic + insulin (24 h)	5	155 ± 3	879 ± 71	1.18 ± 0.24‡	92	2.47 ± 0.29*	0.23 ± 0.05	9.88 ± 1.02‡	2.09

fat-refed rats incubated with insulin showed, in all cases (Tables 6, 7 and 9) a significant increase in the lactate/pyruvate ratio, as also did tissues from animals fed a high-fat diet without preliminary starvation (Table 7). The medium [lactate]/[pyruvate] ratio was not significantly elevated in the case of alloxan-diabetic tissues (Table 7), but was significantly elevated if the donor diabetic animals had been pretreated with insulin for 24h before incubation of tissues. Incubation of whole or segmented tissues from normal, starved, fat-refed, or starved, bread-refed rats with acetate in the presence of insulin considerably decreased the medium [lactate]/[pyruvate] ratio compared with the same tissues incubated with insulin alone (Tables 6 and 9). However, no significant alterations in this ratio with starved animals were observed in the presence of acetate.

Values are presented in Table 6 for the extramitochondrial free [NAD^+]/[NADH] ratios in the incubated tissues as calculated independently from the medium [lactate]/[pyruvate] ratio (making the arbitrary assumption that the ratio in the incubation medium at the end of the incubation is the same as that in the tissues at the time of sampling) and from the [glycerol 1-phosphate]/[dihydroxyacetone phosphate] ratio. Reasonable agreement, both in terms of the absolute values of the ratios, and of the direction of change of these ratios in the presence of sodium acetate, is obtained with tissues from starved and starved, fat-refed rats. In the case of the tissues from starved, bread-refed rats however, there is considerable disagreement between the two estimates as to the direction of change of the [NAD^+]/[NADH] ratio on incubation with acetate. Also the agreement between the estimates of this ratio for normal tissues is poor, although both methods of assessment indicated that the extramitochondrial [NAD^+]/[NADH] ratio in normal tissues incubated with insulin+acetate is more oxidized than that in normal tissues incubated with insulin alone (Saggerson & Greenbaum, 1970).

Rates of whole-tissue fatty acid synthesis from glucose in vitro *and activities of fatty acid synthesizing enzymes in adipose tissues from rats of varied dietary status.* The results in Table 7 indicate that [^{14}C]-glucose incorporation into fatty acids by segments of adipose tissue *in vitro* is severely curtailed by starvation. On refeeding bread to the donor animals, the ability to incorporate glucose into fatty acid is regained and becomes greater than normal after 144h of refeeding. On the other hand, the refeeding of a high-fat diet after starvation did not appear to elicit an appreciable restoration of fatty acid synthesis. Feeding a high-fat diet and induction of alloxan-diabetes also led to considerably diminished capacities of the tissues to synthesize fatty acids from glucose. Insulin therapy of alloxan-diabetic animals for 24h partially restored the fatty acid synthesizing capacity of the tissues. Although not shown in Table 7, the incorporation of [^{14}C]glucose into glyceride glycerol and non-saponified lipids was also routinely monitored, and the total radioactivity incorporated into these two fractions and into the fatty acid fraction was compared with the incorporation into total lipids, which was also monitored. Table 7 shows that the total percentage recovery of lipid fractions on saponification and extraction was good (>90%) except in the experiment with alloxan-diabetic tissues.

Table 8 shows that both the total fat pad activities and the activities per mg of tissue N of acetyl-CoA carboxylase and fatty acid synthetase were considerably decreased in tissues from starved and alloxan-diabetic rats. The concentrations in tissues from starved rats were restored by refeeding bread over a period of up to 4 days, but not by refeeding the high-fat diet. These considerable fluctuations in activity may be compared with the noticeable constancy of the activity per mg of tissue N of isocitrate dehydrogenase (NADP) which was routinely assayed with the other two enzymes. It was decided that for comparison of enzyme activities between the adipose tissues from rats of varied dietary status and fat content, the expression of activities on a nitrogen basis was more suitable than expression of activities per pair of fat pads or per g wet wt. of tissue.

Measurements of carbon fluxes through metabolic pathways in incubated segments of adipose tissues. In Tables 9 and 10 measurements are presented of the rates of flux of glucose-derived carbon through various metabolic pathways in the presence and absence of acetate. The tables also indicate the magnitude of the flux of acetate-derived carbon into CO_2 and into fatty acids in the presence of glucose. It may be seen that in tissues from starved and starved, fat-refed rats glucose utilization by the glycolytic pathway, and more particularly by the hexose monophosphate pathway, is considerably decreased below the normal level. In tissues from starved, bread-refed rats on the other hand, glucose utilization by both pathways was above normal. Rates of citric acid cycle CO_2 formation from acetate or from glucose did not, however, appear to be affected by the type of dietary manipulation of the donor animal (Table 10). In all cases, addition of acetate to the incubation medium led to an increased rate of citric acid cycle CO_2 production from glucose. As shown in Table 7, previous starvation or starvation and fat-refeeding of the donor animals led to greatly diminished rates of fatty acid synthesis from glucose. Table 10 shows that fatty acid synthesis from acetate was also diminished under these conditions. On the other hand, rates of fatty acid synthesis

Table 8. *Variation of activities of epididymal fat pad acetyl-CoA carboxylase, fatty acid synthetase and isocitrate dehydrogenase (NADP) with changes in dietary and insulin status*

Treatment of animals and assay of enzyme activities were as described in the Materials and Methods section. The activities of acetyl-CoA carboxylase represent μmol of $H^{14}CO_3^-$ incorporated into acid-stable material/h. The activit es of fatty acid synthetase and isocitrate dehydrogenase (NADP) represent utilization or production in μmol of NADPH/h respectively. The results are mean values ± S.E.M. *$P<0.05$; †$P<0.01$; ‡$P<0.001$ (versus the normally fed control).

	Activity (per pair of fat pads)			Activity (per mg of tissue N)					
Dietary status of animals	Acetyl CoA carboxylase	Fatty acid synthetase	Isocitrate dehydrogenase (NADP)	Acetyl-CoA carboxylase	Fatty acid synthetase	Isocitrate dehydrogenase (NADP)	No. of animals	Mean body wt. of animals (g)	Mean total wt. of fat pads (mg)
Normal	8.29 ± 1.01	20.9 ± 2.9	35.4 ± 2.4	2.55 ± 0.44	6.13 ± 0.98	10.36 ± 1.20	22	178 ± 4	1385 ± 109
Starved for 72h	2.39 ± 0.55‡	3.9 ± 0.9‡	22.3 ± 1.7‡	0.86 ± 0.14‡	1.44 ± 0.25‡	8.63 ± 1.75	7	114 ± 4	485 ± 31
Starved for 72h and refed bread for 24h	4.18 ± 0.73†	6.9 ± 0.7‡	25.7 ± 1.7†	1.36 ± 0.18*	2.16 ± 0.14‡	8.27 ± 0.70	6	142 ± 2	643 ± 77
Starved for 72h and refed bread for 48h	2.96 ± 0.38‡	18.8 ± 1.4	27.7 ± 2.7*	1.16 ± 0.14‡	7.61 ± 1.03	10.90 ± 1.46	7	140 ± 2	687 ± 54
Starved for 72h and refed bread for 72h	6.65 ± 0.92	27.5 ± 1.3*	24.5 ± 2.4†	2.09 ± 0.27	8.62 ± 0.57*	7.66 ± 0.84	7	147 ± 3	716 ± 112
Starved for 72h and refed bread for 96h	11.43 ± 1.47	35.3 ± 3.6†	35.1 ± 2.8	3.69 ± 0.70	11.23 ± 1.90*	11.16 ± 1.82	10	153 ± 3	1327 ± 151
Starved for 72h and refed high-fat diet for 72h	2.60 ± 0.25‡	6.7 ± 0.5‡	36.7 ± 3.6	0.86 ± 0.12‡	2.25 ± 0.28†	12.63 ± 2.45	7	142 ± 3	1301 ± 94
Alloxan-diabetic	1.08 ± 0.20‡	2.4 ± 0.3‡	14.2 ± 2.9‡	0.65 ± 0.18‡	1.61 ± 0.45‡	8.32 ± 2.24	6	130 ± 3	349 ± 134

Table 9. *Measurements of fluxes of carbon derived from glucose through various metabolic pathways in incubated segments of epididymal adipose tissues obtained from rats of varied dietary status*

Treatment of animals, incubation of tissues and calculation of flux rates were as described in the Materials and Methods section. The results are presented as μg-atoms of carbon/h per mg of tissue N and are mean values ± S.E.M. All tissues incubated with insulin alone are compared with tissues from normal animals incubated with insulin alone *$P<0.05$; †$P<0.01$; ‡$P<0.001$. Tissues from rats of varied dietary status incubated with insulin + acetate are compared with tissues from rats of the same dietary status incubated with insulin alone. §$P<0.05$; ||$P<0.01$.

Expt. no.	Dietary status of animals	Mean body wt. of animals (g)	No. of determinations	Additions to incubation medium	Hexose monophosphate pathway CO_2 production	Hexose phosphate to triose phosphate flux	Glyceride-glycerol formation	Triose phosphate to pyruvate flux	Lactate + pyruvate release	$\frac{[Lactate]}{[Pyruvate]}$ ratio in the incubation medium
5	Normal	163 ± 3	8	Insulin	3.07 ± 0.47	20.20 ± 2.73	1.59 ± 0.17	21.70 ± 3.05	4.13 ± 0.35	7.84 ± 0.72
			8	Insulin + acetate	4.80 ± 0.76	26.80 ± 4.65	1.91 ± 0.24	29.70 ± 5.16	4.56 ± 0.20	4.65 ± 0.39||
6	Starved for 72 h	119 ± 4	5	Insulin	0.74 ± 0.23‡	11.98 ± 2.08*	1.69 ± 0.29	11.03 ± 1.94*	8.56 ± 1.69*	7.71 ± 0.44
			5	Insulin + acetate	1.02 ± 0.41	12.75 ± 2.15	1.68 ± 0.33	12.10 ± 2.24	7.26 ± 1.20	7.02 ± 0.39
7	Starved for 72 h and refed high-fat diet for 72 h	148 ± 5	5	Insulin	1.17 ± 0.08†	10.38 ± 1.84*	1.31 ± 0.15	10.24 ± 1.74†	7.58 ± 1.19*	13.00 ± 1.25†
			5	Insulin + acetate	1.41 ± 0.27	7.24 ± 0.64	1.22 ± 0.13	7.43 ± 0.79	4.33 ± 0.43§	10.28 ± 0.69
8	Starved for 72 h and refed bread for 144 h	152 ± 7	5	Insulin	6.28 ± 0.83†	36.30 ± 5.30*	2.42 ± 0.31*	41.17 ± 5.83*	8.79 ± 1.42†	4.94 ± 0.28†
			5	Insulin + acetate	10.38 ± 1.28§	42.68 ± 5.90	2.65 ± 0.38	50.39 ± 6.79	13.11 ± 2.03	3.57 ± 0.30||

Table 10. *Measurements of fluxes of carbon derived from glucose and acetate through various metabolic pathways in incubated segments of epididymal adipose tissues (continued)*

Treatment of animals, incubation of tissues and calculation of flux rates were as described in the Materials and Methods section. The results are presented as μg-atoms of carbon/h per mg of tissue N and are mean values ± S.E.M. *$P<0.05$, †$P<0.01$ and ‡$P<0.001$ when results of an incubation are compared with those of an identical incubation of tissues from animals of normal dietary status. §$P<0.05$ and ||$P<0.001$ when incubations of tissues of varied dietary status with insulin + acetate are compared with incubations of tissues of the same dietary status with insulin alone.

Expt. no.	Dietary status of animals	Additions to incubation medium	CO_2 produced by pyruvate decarboxylation	CO_2 produced by citric acid cycle (derived from glucose carbon)	CO_2 produced by citric acid cycle (derived from glucose + acetate carbon)	Fatty acid synthesis from glucose	Fatty acid synthesis from glucose + acetate	$\frac{[Glucose]}{[acetate]}$ ratio in citric acid cycle CO_2	$\frac{[Glucose]}{[acetate]}$ ratio in fatty acids	Glycerol release (μg-atoms of carbon)
5	Normal	Insulin	5.85 ± 1.00	0.25 ± 0.03	—	11.45 ± 1.97	—	—	—	0.69 ± 0.12
		Insulin + acetate	8.35 ± 1.70	0.82 ± 0.13||	1.24 ± 0.14	15.95 ± 3.30	18.66 ± 3.59	1.98	5.88	0.60 ± 0.10
6	Starved for 72 h	Insulin	0.82 ± 0.16‡	0.31 ± 0.05	—	1.34 ± 0.31‡	—	—	—	1.35 ± 0.24*
		Insulin + acetate	1.62 ± 0.42	0.59 ± 0.10§	1.03 ± 0.22	2.65 ± 0.80	3.27 ± 0.96†	1.34	4.35	1.11 ± 0.09
7	Starved for 72 h and refed high-fat diet for 72 h	Insulin	0.89 ± 0.27†	0.39 ± 0.08	—	1.39 ± 0.45‡	—	—	—	1.23 ± 0.27
		Insulin + acetate	1.03 ± 0.18	0.48 ± 0.01	1.13 ± 0.10	1.59 ± 0.37	2.30 ± 0.43‡	0.74	2.26	1.02 ± 0.15
8	Starved for 72 h and refed bread for 144 h	Insulin	10.79 ± 1.51*	0.30 ± 0.03	—	20.92 ± 3.29*	—	—	—	0.84 ± 0.15
		Insulin + acetate	12.43 ± 1.64	0.58 ± 0.08§	0.93 ± 0.13	24.28 ± 3.25	28.12 ± 3.71	1.89	6.40	0.97 ± 0.12

from glucose and from acetate were greater than normal in tissues from starved, bread-refed rats. In all cases the inclusion of acetate in the incubation medium elicited increased rates of fatty acid synthesis from glucose together with increased rates of hexose monophosphate pathway CO_2 production and, in the case of tissues from normal, starved and starved, bread-refed rats, increased rates of glucose utilization by glycolysis. With tissues from starved, fat-refed rats however, the rate of glucose utilization by glycolysis was in fact decreased when acetate was added and instead, more glucose carbon became available for fatty acid synthesis through a decrease in the rate of release of lactate+pyruvate.

Table 10 also shows that the ratios of glucose carbon to acetate carbon appearing in the products (fatty acids and citric acid cycle CO_2) were less in the case of tissues from starved and starved, fat-refed rats than in those from normal and starved, bread-refed rats, suggesting that in tissues from starved and starved, fat-refed animals glucose is discriminated against, compared with acetate, as a supplier of acetyl-CoA. It is also noteworthy that the glucose carbon/acetate carbon ratio was found to be much higher in fatty acids than in citric acid cycle CO_2 in all four experimental groups.

DISCUSSION

From the results presented above, it is apparent that previous fasting, fasting and fat-refeeding, fat-feeding and induction of alloxan diabetes in rats all bring about a lesion in fatty acid synthesis from glucose *in vitro* in tissues taken from these animals. The results presented may offer a partial explanation of this lesion.

Activities of acetyl-CoA carboxylase and fatty acid synthetase. Although the rat adipose tissue acetyl-CoA carboxylase and fatty acid synthetase have been purified and extensively characterized by Martin & Vagelos (1962), Vagelos *et al.* (1963), Martin, Horning & Vagelos (1961) and Horning, Martin, Karmen & Vagelos (1961) there appear, except for a paper by Dakshinamurti & Desjardins (1969), to be no reports in the literature concerning variations of the activities of these two enzymes with physiological state in adipose tissue.

It is probably not valid to compare in a quantitative manner the maximal activities of these enzymes as measured in the extracts prepared in the present study (Table 8) with measured rates of fatty acid synthesis *in vivo* or in intact tissues *in vitro*. This is particularly true for acetyl-CoA carboxylase. This enzyme appears to have such a complex set of substrate and activator requirements that, at present, it is not possible to assess whether any combination of these *in vitro* would give an activity approaching maximal. It should be stressed that several factors, not included in the assays recorded in Table 8, alter the rate of the acetyl-CoA carboxylase reaction. For example, there are several reports of liver cytoplasmic particles activating or inhibiting soluble preparations of liver and adipose tissue acetyl-CoA carboxylase (Masoro, Korchak & Porter, 1962; Korchak & Masoro, 1963; Howard & Lowenstein, 1964; Martin & Pittman, 1966), and in fact Margolis & Baum (1966) have suggested that pigeon liver acetyl-CoA carboxylase may be associated with membrane material in the cell. The possible complexity of the behaviour of acetyl-CoA carboxylase has also been indicated by the findings of Gregolin, Ryder, Warner, Kleinschmidt & Lane (1966), Greenspan & Lowenstein (1967) and Fang & Lowenstein (1967). Comparison of the measured enzyme activities with whole-tissue rates of fatty acid synthesis is also complicated by the fact than an appreciable, but unknown, proportion of the tissue fatty acid synthesizing capacity, in liver at least, appears to reside in the mitochondrial and microsomal fractions of centrifuged tissue homogenates. The results presented in this study refer only to measurements made on high-speed supernatants. However, the far higher dilutions used in preparation of adipose tissue homogenates compared with liver homogenates may act to remove enzymes adhering to membrane structures (Margolis & Baum, 1966). Hubbard, McCaman, Smith & Gibson (1961) have demonstrated the existence of a [1,3-^{14}C]malonyl-CoA decarboxylase in rat liver high-speed supernatant preparations. This activity was not greatly affected by starving and refeeding. If a similar activity is present in adipose tissue, the results for acetyl-CoA carboxylase shown in Table 8 may well be an underestimation of the activity of that enzyme, particularly in those physiological states where the activity is lowest. It should also be pointed out that phosphorylated sugar activators of fatty acid synthetase (Wakil, Goldman, Williamson & Toomey, 1966) were not included in the assay of that enzyme complex in this study. However, the assay was performed in an orthophosphate buffer which is itself a slight activator.

Although the measured activities of acetyl-CoA carboxylase and fatty acid synthetase cannot be quantitatively correlated with other physiological parameters, it is probably reasonable to attempt qualitatively to correlate changes in the measured activities of these two enzymes with other physiological changes. The results of this study suggested that the adipose tissue acetyl-CoA carboxylase and fatty acid synthetase were quite adaptive in response to changes in dietary status, and far more so than the isocitrate dehydrogenase, which was assayed in the same extracts as a non-adaptive

control (Young *et al.* 1964; Leveille & Hanson, 1966) and also more so than several adipose tissue glycolytic enzymes (Saggerson & Greenbaum, 1969). The alteration of the adipose tissue contents of acetyl-CoA carboxylase and fatty acid synthetase in response to starvation, alloxan-diabetes and high-carbohydrate refeeding appeared to be qualitatively the same as the responses of the liver enzymes to such treatments (Gibson & Hubbard, 1960; Hubbard, McCaman *et al.* 1961; Numa *et al.* 1961; Wieland *et al.* 1962; Korchak & Masoro, 1962; Bortz *et al.* 1963; Allman *et al.* 1965), although the responses of the adipose tissue enzymes under the conditions used in this study did not appear to be as swift, or of such a magnitude, as those of the liver. The failure in adipose tissue of rats fed the high-fat diet (mainly unsaturated fatty acid) to restore the acetyl-CoA carboxylase and fatty acid synthetase contents after starvation has also been observed in liver (Allman *et al.* 1965). It is noteworthy, however, that administration of the high-fat diet could restore the content of isocitrate dehydrogenase (NADP) (Table 8) and the contents of several glycolytic enzymes (Saggerson & Greenbaum, 1969).

In general, alterations in the measured activities of adipose tissue acetyl-CoA carboxylase and fatty acid synthetase in response to dietary and hormonal manipulation (Table 8) paralleled alterations in the ability of tissues from similarly treated rats to incorporate glucose or acetate into fatty acids *in vitro* (Tables 7 and 10) in that starvation and induction of alloxan-diabetes resulted in a lowering of the activities of the two enzymes and of the rate of fatty acid synthesis *in vitro*. Refeeding with bread after starvation resulted in an increase in these three parameters, whereas refeeding with the high-fat diet did not. A similar parallelism has been drawn between the lipogenic capacity of adipose tissues *in vitro* and their contents of malate dehydrogenase (NADP) and ATP–citrate lyase (Wise & Ball, 1964; Young *et al.* 1964; Kornacker & Ball, 1965; Brown & McLean, 1965). However, the demonstration of such a parallelism does not, by itself, indicate whether altered rates of lipogenesis *in vitro* are wholly or partially dependent on, or independent of, altered enzyme contents of the adipose tissues. In rat liver there does not appear to be any correlation between the resumption of lipogenesis *in vitro* and the adaptive response of lipogenic enzymes on refeeding a high-carbohydrate, protein-free diet after starvation (Tepperman, De la Garza & Tepperman, 1968). Srere & Foster (1967) have also demonstrated a lack of correlation between the liver content of ATP–citrate lyase and the lipogenic capacity of liver slices. However, Jomain & Hanson (1969) have demonstrated that in adipose tissue, unlike liver, low rates of lipogenesis *in vitro* are accompanied by lowered contents of malate dehydrogenase (NADP), ATP–citrate lyase and hexose monophosphate pathway dehydrogenases in tissues from animals refed a high-carbohydrate, protein-free diet. In adipose tissue therefore, the significance to be attached to prior alterations in enzyme contents in explaining altered rates of lipogenesis *in vitro* is unclear.

Correlations of whole-tissue metabolite concentrations with carbon fluxes. In normal adipose tissues incubated in the presence of glucose together with other agents that lead to alterations in the rates of triglyceride and fatty acid synthesis there appears to be no meaningful correlation between these parameters and the concentration in the tissues of metabolites such as citrate, long-chain fatty acyl-CoA and glycerol 1-phosphate (Denton & Halperin, 1968; Saggerson & Greenbaum, 1970). Similar correlations for the results obtained in this study were attempted (Table 11). Denton & Halperin (1968) pointed out that in attempting to draw conclusions from such correlations it is necessary to assume that changes in whole-tissue concentrations of metabolites reflect changes in relevant cell compartments. The validity of this assumption cannot be easily established. In Table 11 the metabolite concentrations are, in fact, presented per mg of tissue N. However, it would seem to be as valid to correlate these values with the flux rates as to correlate whole-tissue concentrations since significant alterations in the volume of intracellular water per mg of tissue N were not encountered between the dietary treatments (Table 5).

Table 11 shows that rates of triglyceride synthesis were essentially similar in all the dietary conditions studied. No conclusions concerning the control of fatty acid esterification can therefore be drawn from correlations of these flux rates with the tissue concentrations of long-chain fatty acyl-CoA and glycerol 1-phosphate. In fact, as was noted by Saggerson & Greenbaum (1970), under the conditions used in this study, i.e. high, non-physiological concentrations of glucose and insulin in the incubation media, the fatty acid esterifying system of the tissue is probably always near to saturation with glycerol 1-phosphate. Whole-tissue concentrations of this metabolite, from the results of Tables 2 and 5 of the present study, ranged from 2.1mM for tissues from normal rats to 5.0mM for tissues from starved, fat-refed rats. Also, the concentrations of glycerol 1-phosphate in the tissues did not appear to be related to the glycolytic rate. However, the extramitochondrial free $[NAD^+]/[NADH]$ ratio is probably of importance in governing the concentration of this metabolite (Halperin & Denton, 1969; Saggerson & Greenbaum, 1970) since the tissues from starved, fat-refed rats contained the highest concentrations of glycerol 1-phosphate and were

Table 11. *Relationship between the rates of carbon flux through various metabolic stages and the concentrations of metabolic intermediates*

The metabolite measurements were taken from Tables 2 and 4 and the flux measurements from Tables 9 and 10. The results refer only to tissues incubated with glucose+insulin and are expressed as percentages of the values observed with normal tissues.

Dietary status of animals	Metabolite concentrations (per mg of tissue N(as % of concentrations in normal tissues					Flux rates (per mg of tissue N) as % of concentrations in normal tissues				
	Long-chain fatty acyl-CoA	Acid-soluble CoASH	G6P	G1P	Citrate	Triglyceride synthesis	Hexose phosphate to triose phosphate	Pyruvate+lactate release	Pyruvate decarboxylation	Fatty acid synthesis
Normal	100	100	100	100	100	100	100	100	100	100
Starved for 72h	106	43	75	114	241	106	59	207	14	12
Starved for 72h and refed high-fat diet for 72h	79	101	119	253	336	82	51	184	15	12
Starved for 72h and refed bread for 144h	127	163	137	122	141	152	185	213	184	183

also associated with the highest [lactate]/[pyruvate] ratios in the incubation medium (Tables 1, 7 and 9). In this study the rate of triglyceride synthesis was calculated from the rate of flow of [U-^{14}C]glucose-derived carbon into glyceride glycerol. The validity of this approach has been discussed by Denton & Randle (1967) and by Denton & Halperin (1968).

Considerable variations in the flux of glucose carbon into fatty acids were encountered in the treatments studied. However, these cannot be meaningfully related to the proposed roles of long-chain fatty acyl-CoA as an inhibitor of fatty acid synthesis or of citrate as an activator. Concentrations of long-chain fatty acyl-CoA were remarkably constant in the four cases studied, although their proportion of the measured CoA pool (total CoA) varied considerably in the four physiological states. The apparent depletion of the total CoA pool on starvation and its replenishment on refeeding is a notable observation. Considerable variations in the concentrations of citrate were observed; however, the highest concentrations of citrate were encountered in tissues with the lowest rates of fatty acid synthesis. Denton *et al.* (1966) reported elevated citrate concentrations in tissues from alloxan-diabetic rats incubated in the presence of glucose+insulin. Ballard & Hanson (1969) have also recorded the absence of any direct relationship between the total citrate concentration in epididymal fat pads *in vivo* and rates of fatty acid synthesis. It is possible, however, as discussed by Saggerson & Greenbaum (1970), that acetyl-CoA carboxylase may be saturated with citrate at the concentrations present in these tissues. It should be pointed out that in assessment of the rate of fatty acid synthesis it is assumed that the specific activity of the immediate fatty acid precursor, acetyl-CoA, is the same as that of glucose. Since adipose tissues appear to be able to metabolize free fatty acids at appreciable rates under certain circumstances (Milstein & Driscoll, 1959; Bally, Cahill, Le Boeuf & Renold, 1960), dilution of this acetyl-CoA pool might be expected, particularly in the starved and starved, fat-refed states where the rate of endogenous lipolysis, as indicated by glycerol release, is increased. However, Tables 9 and 10 indicate that the rates of glyceride-glycerol formation in all four dietary states generally slightly exceed the rates of formation required to re-esterify all the fatty acids released by endogenous lipolysis (assuming three fatty acid molecules released/glycerol molecule) and the fatty acids synthesized *de novo* from glucose (assuming an average chain length of 17 carbon atoms; Flatt & Ball, 1964). In this case dilution of the fatty acid precursor pool may not lead to serious underestimation of the rate of fatty acid synthesis, although any error introduced by the metabolism

of fatty acids present in the tissue at the commencement of incubation cannot be assessed.

Table 11 indicates that the low rates of glucose-carbon flux to fatty acids in tissues from starved and starved, fat-refed rats are not accompanied by decreased contents of glucose 6-phosphate. Table 2 shows that the size of the tissue content of glucose 6-phosphate is indicative of the content of the other glycolytic intermediates measured. When tissues from normal animals are incubated with glucose in the absence of insulin, or in the presence of anti-insulin serum, tissue contents of glycolytic intermediates are depleted compared with insulin-treated tissues and the fluxes of glucose-carbon through the glycolytic pathway and into fatty acids are decreased to approximately the same extent (Denton *et al.* 1966; Halperin & Denton, 1969; Saggerson & Greenbaum, 1970). The absence of such a depletion of glycolytic intermediates, and the fact that glycolytic flux is not decreased by an amount proportional to the decrease in the rate of fatty acid synthesis in tissues from starved and starved, fat-refed rats suggests that, unlike normal tissues incubated without insulin, the lesion in fatty acid synthesis cannot be explained by a decreased ability to transport glucose.

Although not of the same magnitude as the decrease in fatty acid synthesis, the approximate halving in the flux of glucose-derived carbon from hexose phosphate to triose phosphates in tissues from starved and starved, fat-refed rats would indicate the possibility of metabolic control at the phosphofructokinase level. A diminished activity of phosphofructokinase under these conditions would correlate with the observed increases in the concentrations of citrate, a known inhibitor of adipose tissue phosphofructokinase (Denton & Randle, 1966). That phosphofructokinase may be capable of regulatory behaviour in the tissues in this study is indicated by the fact that the mass-action ratio for this reaction is considerably less than the apparent equilibrium constant (Table 6). Evidence for regulatory behaviour of phosphofructokinase in normal adipose tissues *in vitro* has been obtained by Halperin & Denton (1969) and by Saggerson & Greenbaum (1970). Unfortunately, since the contents of glycolytic enzymes are different in the various physiological states under study here (Saggerson & Greenbaum, 1969), correlations between alterations in the tissue concentrations of fructose 6-phosphate and alterations in the rate of glycolytic flux cannot be made in attempts to clarify the possibility of phosphofructokinase regulation through citrate inhibition. Such an inhibition would however, only partially explain the lesion in fatty acid synthesis occurring in tissues from starved and starved, fat-refed rats. Other possible points of metabolic regulation governing the synthesis of fatty acids from triose phosphates are considered below. It should be pointed out that citrate regulation of phosphofructokinase is not consistent with the elevated concentrations of citrate and the increased glycolytic flux in tissues from starved, bread-refed rats unless other factors are involved. Presumably the elevated steady-state concentrations of citrate in tissues from starved and starved, fat-refed animals reflect a decrease in the ability of tissues to utilize citrate relative to its synthesis, thus establishing a new steady state. The reasons for a decreased ability to utilize citrate in these tissues is unclear; however, the fact that tissues from starved and starved, fat-refed rats contain decreased contents of ATP–citrate lyase, acetyl-CoA carboxylase and fatty acid synthetase, as discussed above, should be borne in mind. A control mechanism whereby the cytoplasmic concentration of citrate regulates the activity of phosphofructokinase so that its production keeps pace with its utilization would appear to be reasonable, but would not be consistent with the proposed role of citrate as an activator of acetyl-CoA carboxylase unless there were a considerable difference between the cytoplasmic concentrations at which citrate exerted its effects on phosphofructokinase and acetyl-CoA carboxylase. Citrate regulation of phosphofructokinase would presumably be transmitted to glucose uptake via a glucose 6-phosphate-mediated inhibition of adipose tissue hexokinases (Grossbard & Schimke, 1966), thus accounting for the observations that glucose uptake *in vitro* is low in tissues from diabetic, starved and starved, fat-refed rats (Denton *et al.* 1966; E. D. Saggerson & A. L. Greenbaum, unpublished work).

Enzyme mass-action ratios. As was considered above, the results in Table 6 indicate that in all the experimental conditions studied, the phosphofructokinase reactants appear to be considerably displaced from equilibrium (Hess, 1963) and this displacement is similar in each dietary state. In all cases the reactants of phosphoglucose isomerase were very close to their equilibrium values, as found in other tissues (Hess, 1962; Lowry & Passonneau, 1964; Williamson, 1965; Minakami & Yoshikawa, 1966; Rolleston & Newsholme, 1967) and in normal adipose tissues over a range of incubation conditions (Saggerson & Greenbaum, 1970). This near-equilibration of the phosphoglucose isomerase reactants is not unexpected since the activity of this enzyme is high in all the physiological states studied (Saggerson & Greenbaum, 1969). The mass-action ratio for the combined reaction aldolase $\times$ triose phosphate isomerase was usually one-fifth to one-tenth of the product of the apparent equilibrium constants for the two reactions, which varies between 1.4×10^{-3} M and 3.7×10^{-3} M, depending on the combination of

published apparent equilibrium constants used (Herbert *et al.* 1940; Meyerhof & Junowicz-Kocholaty, 1943; Krebs & Kornberg, 1957; Hess, 1963; Lowry & Passonneau, 1964). In most cases the Γ values for the separate aldolase and triose phosphate isomerase reactions could not be ascertained since the tissue concentrations of glyceraldehyde 3-phosphate were too low to measure. However, reasonable estimates of the concentration of this metabolite were made in tissues from starved, bread-refed rats and the results indicated that, for incubated tissues of this dietary group at least, the aldolase reactants were present in near-equilibrium proportions, whereas those of the triose phosphate isomerase reaction were not ($K_a/\Gamma \simeq 5$).

Effects of acetate on glucose metabolism. In all four dietary states the addition of acetate led to increased total rates of fatty acid synthesis. In tissues from normal and starved rats the increases were 63% and 144% respectively, results very similar to those reported by Flatt & Ball (1966). In tissues from starved, fat-refed and starved, bread-refed rats the increases were 65% and 34% respectively. These results suggest that the observed rates of fatty acid synthesis in the presence of glucose alone in all four dietary states are not governed by the altered maximal activities of lipogenic enzymes. However, in tissues from starved and starved, fat-refed rats, the total rates of fatty acid synthesis and the rates of synthesis solely from acetate were greatly diminished compared with those in normal tissues whereas those in tissues from starved, bread-refed rats were elevated. Whether altered enzyme contents contribute to these differences cannot be ascertained. In all four dietary states the increases in fatty acid synthesis in the presence of acetate were accompanied by increases in the calculated flux of glucose-carbon through the hexose monophosphate pathway, suggesting that altered rates of fatty acid synthesis accompanying dietary manipulations cannot be accounted for by the possibility that altered contents of the hexose monophosphate pathway dehydrogenases (Hollifield & Parson, 1965; Leveille & Hanson, 1966; McLean, Brown & Greenbaum, 1968) limit the supply of NADPH.

In all four dietary states the flux of glucose-carbon into citric acid cycle CO_2 was slightly increased in the presence of acetate, and three- to five-fold increases in the total flux of glucose + acetate carbon into citric acid cycle CO_2 were observed. Similar increases in measured citric acid cycle flux in the presence of acetate in normal tissues were reported by Rognstad & Katz (1966). This effect was not observed by Flatt & Ball (1966). It should be stressed that the citric acid cycle results of this study and of Rognstad & Katz (1966) do not include any assessment of the production of citric acid cycle CO_2 from endogenous precursors. The fact that the incorporation of acetate relative to glucose into citric acid cycle CO_2 is better than into fatty acids is apparent from Table 10. In each dietary state there is a difference of approximately threefold between the glucose carbon/acetate carbon ratios for citric acid cycle CO_2 and fatty acids. A result of this nature would suggest the existence of mitochondrial and extramitochondrial pools of acetyl-CoA of different specific radioactivities when glucose and [^{14}C]acetate are used together as substrates. A bimodal intracellular localization of adipose tissue acetyl-CoA synthetase, as found in liver by Aas & Bremer (1968) might account for the results of this study. The results presented here are, however, not consistent with those presented by Rognstad & Katz (1966) or by Flatt & Ball (1966). It is also necessary to consider the possibility that $^{14}CO_2$ is produced from [U-^{14}C]acetate by pathways other than the citric acid cycle. Leveille (1967) has demonstrated incorporation of [^{14}C]acetate into glyceride glycerol in the presence of pyruvate. $^{14}CO_2$ would be produced in this pathway by the phosphoenolpyruvate carboxykinase reaction. However, in the presence of glucose, [^{14}C]acetate incorporation into glyceride glycerol appears to be very small. The glucose carbon/acetate carbon ratios in citric acid cycle CO_2 and in fatty acids are decreased in tissues from starved and starved, fat-refed rats, indicating that there may be a metabolic block in these tissues between the uptake of glucose and the formation of acetyl-CoA compared with tissues from normal and starved, bread-refed rats. It is noteworthy that Haft (1964) has reported a similar discrimination against glucose compared with acetate as a lipogenic precursor in perfused hearts from diabetic rats. In general, the altered metabolic rates in the presence of acetate were not accompanied by significant alterations in the tissue concentrations of metabolites (Tables 2, 3 and 4). No conclusions concerning the nature of the effect of acetate can therefore be drawn from these measurements.

Metabolism of pyruvate and its dependence on endogenous lipolysis. Table 11 shows that although rates of fatty acid synthesis from glucose were greatly decreased in tissues from starved and starved, fat-refed rats, glycolytic rates were not correspondingly altered. In these tissues pyruvate decarboxylation was, however, decreased to approximately the same extent as fatty acid synthesis while lactate + pyruvate release was disproportionately high. In tissues from starved, bread-refed rats the glycolytic rate, lactate + pyruvate release, pyruvate decarboxylation and fatty acid synthesis were all elevated to approximately the same extent. The results indicate that in tissues from starved and starved, fat-refed rats the diminution in fatty

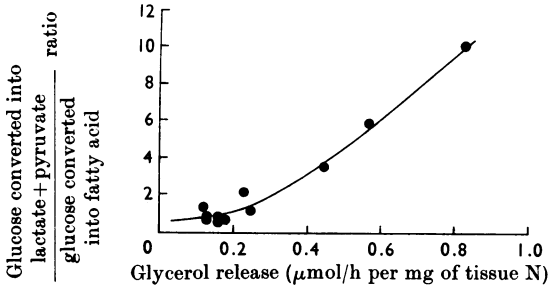

Fig. 1. Relationship between the rate of release of glycerol and the relative rates of production of lactate + pyruvate and fatty acids by incubated adipose tissues from rats of varied dietary status.

acid synthesis rate is to some extent brought about by a decreased ability of the tissues to decarboxylate pyruvate. Table 7 suggests that this situation also holds for tissues subjected to other dietary and hormonal manipulation as indicated by the high value of the lactate+pyruvate released/fatty acid synthesis ratio in some, but not all, tissues exhibiting low rates of lipogenesis. In all cases Table 7 shows that high values of this ratio were encountered in tissues displaying an elevated rate of endogenous lipolysis (as monitored by glycerol release). A relationship between the relative utilization of glucose carbon for lactate+pyruvate formation or fatty acid synthesis and free fatty acid release within the tissue is illustrated in Fig. 1. Although this relationship may be fortuitous, it may have metabolic significance. Presumably free fatty acids released inside the adipose tissues by breakdown of tissue glycerides will either be re-esterified or metabolized by the tissue. Under the conditions employed in this study, release of free fatty acids into the surrounding medium can be discounted since, in order to demonstrate free fatty acid release from adipose tissue *in vitro*, albumin must be present in the incubation medium (Reshef, Shafrir & Shapiro, 1958). Adipose tissues influenced by factors such as prior fasting, alloxan-diabetes or treatment with lipolytic hormones are known to have elevated intracellular concentrations of free fatty acids (White & Engel, 1958; Dole, 1961; Vaughan, 1961; Buckle, 1963). Whether increased intracellular concentrations of free fatty acids are accompanied by increased rates of their metabolism is unknown, although Bally *et al.* (1960) have shown that oxidation of palmitate to CO_2 by adipose tissues *in vitro* is linearly related to its concentration in the incubation medium in the absence of glucose. It would seem reasonable to speculate that metabolism of fatty acids by adipose tissue would have effects on pyruvate utilization similar to those demonstrated in heart and in liver (Evans, Opie & Renold, 1963; Garland, Newsholme & Randle, 1964; Bremer, 1965, 1966; von Jagow, Westermann & Wieland, 1968). However, the extent to which fatty acids are metabolized by the tissues in this study is unknown. The increased lipolysis of tissue glycerides occurring in normal adipose tissues incubated with adrenaline+insulin compared with tissues incubated with insulin alone is also accompanied by increased rates of lactate+pyruvate release and decreased rates of fatty acid synthesis from glucose (Denton & Halperin, 1968; Saggerson & Greenbaum, 1970). Cahill, LeBoeuf & Flinn (1960) observed that adipose tissues incubated with palmitic acid showed alterations in glucose metabolism similar to those found in tissues incubated with adrenaline and therefore suggested that changes in glucose metabolism observed in the presence of lipolytic hormones may be secondary to elevations of intracellular free fatty acid concentrations resulting from an accelerated lipolysis. Cahill *et al.* (1960), however, did not determine whether incubation with palmitate resulted in altered rates of lactate+pyruvate release. It appears, in fact, that incubation of adipose tissues with palmitate or oleate in the presence of glucose+insulin does not result in elevated rates of lactate+pyruvate release or in decreased rates of fatty acid synthesis from glucose (Denton & Halperin, 1968; Saggerson & Greenbaum, 1970) and only mimics the effect of adrenaline in that glucose metabolism to glyceride glycerol and citric acid cycle CO_2 is increased (Saggerson & Greenbaum, 1970). However, these findings do not necessarily contradict the suggestion that metabolism of fatty acids by adipose tissues may decrease the utilization of pyruvate for fatty acid synthesis, since Dole (1961) showed that, under conditions of incubation similar to those employed in this study i.e. insulin and high concentrations of glucose present, externally supplied ^{14}C-labelled fatty acids do not equilibrate with the intracellular pool(s) of fatty acids. Under the conditions used by Dole (1961) however, considerable esterification of the supplied ^{14}C-labelled fatty acids occurred, suggesting that esterification of medium fatty acids keeps pace with their uptake. These results, and the demonstration by Bally *et al.* (1960) that the oxidation of externally supplied fatty acids by adipose tissues *in vitro* is extremely sensitive to the presence of glucose, suggests that under the conditions used only free fatty acids generated inside the tissue by lipolysis of tissue glycerides may be able to penetrate to the intracellular site(s) of a metabolism that could influence the utilization of pyruvate. The sites of such metabolism should presumably be spatially removed from the sites of fatty acid esterification

within the tissue. The very high tissue concentrations *in vitro* of glycerol 1-phosphate encountered under the incubation conditions used in this study and elsewhere in the presence of insulin (Denton *et al.* 1966; Denton & Halperin, 1968; Saggerson & Greenbaum, 1970) indicates that esterification of externally supplied free fatty acids may be extremely rapid. *In vivo*, however, it is quite possible that esterification of externally supplied free fatty acids may not keep pace with their uptake under certain conditions, thus leading to an elevation of intracellular concentrations of free fatty acids and an increased rate of a mode of their metabolism likely to influence the utilization of pyruvate. Concentrations of glycerol 1-phosphate in adipose tissues freeze-clamped *in situ* and taken from anaesthetized animals are in fact considerably lower than those found *in vitro* (Saggerson & Greenbaum, 1970). A control mechanism may therefore exist *in vivo* that could divert the utilization of carbohydrate sources available to adipose tissues away from fatty acid synthesis and into glycerol 1-phosphate production in response to increased uptake of dietary fatty acids by the tissue. Under the conditions used *in vitro* a control mechanism of this nature may not be apparent due to high potential rates of esterification resulting from the unusually high concentrations of glycerol 1-phosphate unless the effect is mimicked by the intracellular introduction of fatty acids to the tissue through lipolysis of tissue glycerides. At present it is only possible to speculate on the way in which fatty acid metabolism might possibly affect pyruvate utilization in adipose tissues. However, a possible mechanism might involve, as in other tissues, an inhibition of the pyruvate dehydrogenase enzyme complex associated with increased values of the mitochondrial [acetyl-CoA]/[CoASH] and [NADH]/[NAD^+] ratios resulting from increased rates of mitochondrial fatty acid metabolism (Garland & Randle, 1964; Randle *et al.* 1966; Hansen & Henning, 1966; Nicholls, Shepherd & Garland, 1967). Denton, Halperin & Randle (1969) have also considered the possibility that adipose tissue pyruvate dehydrogenase may be subject to a control similar to that found in liver and heart. Further experiments to elucidate the effects of fatty acid metabolism on pyruvate utilization by adipose tissues and adipose tissue mitochondria and to elucidate the control characteristics of adipose tissue pyruvate dehydrogenase are required.

Fate of cytoplasmic NADH. Whatever its mechanism, and whether it is or is not brought about through fatty acid metabolism, any restriction on glucose-derived pyruvate metabolism, as is encountered in tissues from starved, starved, fat-refed, and diabetic rats, will have important metabolic consequences. If oxidative decarboxylation of pyruvate is decreased, less intramitochondrial acetyl-CoA is expected to be available for export to the cytoplasm for fatty acid synthesis, and hence the utilization of NADPH and ATP in that process will be diminished. Flatt & Ball (1966) suggested that a major factor controlling the rate of fatty acid synthesis from glucose is the rate at which NADH produced at the two dehydrogenation steps in the pathway between triose phosphate and acetyl-CoA can be reoxidized. In adipose tissue the major means of reoxidizing this NADH are reductive synthesis of lactate and glycerol 1-phosphate, transhydrogenation via malate dehydrogenase and 'malic enzyme' to yield NADPH for reductive synthesis of fatty acids, and reoxidation by the respiratory chain at a rate presumably coupled to the ATP demand for fatty acid synthesis. Although not fully established, it has been suggested that NADH produced in the cytoplasm of adipose tissue from the oxidation of triose phosphates is used solely in that compartment under normal conditions and that no transfer of hydrogen equivalents from the cytoplasm to the mitochondria takes place (Rognstad & Katz, 1966; Schmidt & Katz, 1969). This is also discussed by Saggerson & Greenbaum (1970). If these concepts are correct, it is most likely that demands for ATP for fatty acid synthesis from glucose, over and above the tissue's basal metabolic needs, will be met by respiration of pyruvate dehydrogenase-derived NADH, the production of which varies in a manner approximately proportional to the supply of acetyl-CoA for fatty acid synthesis through control of pyruvate dehydrogenation. It is of course possible that if fatty acid synthesis were inhibited at some point beyond acetyl-CoA production from pyruvate, mitochondrial NADH might be expected to accumulate due to a decreased demand for ATP for fatty acid synthesis and, through inhibition of pyruvate dehydrogenation, set a new rate of pyruvate utilization. A control mechanism of this nature would not be dependent on the involvement of fatty acid oxidation discussed above and would be consistent with the observed increases in citrate concentrations and depletions in lipogenic enzyme contents in tissues from starved, starved and fat-refed, and diabetic rats. However, a mechanism of this nature would still have to be consistent with the observed increases in lipolytic rate in these tissues, although a direct, inhibitory action of free fatty acids on lipogenic enzymes might explain this. The existence of a block in the lipogenic pathway after pyruvate utilization is supported by the finding in this study that acetate incorporation into fatty acids, though not as seriously decreased as that of glucose, is still low in tissues from starved and starved, fat-refed rats. Also it has been found that acetate incorporation into fatty acids is decreased in tissues incubated

Table 12. *Relative utilization of NADH produced by triose phosphate dehydrogenation for cytoplasmic reductive synthetic purposes*

The results are derived from Table 9. It is assumed, as discussed in the text, that only cytoplasmically produced NADH is used for cytoplasmic reductive syntheses.

Dietary status of animals	Addition to incubation medium	NADH produced by triose phosphate oxidation (μmol/h per mg of tissue N)	% of cytoplasmic NADH used for lactate synthesis	% of cytoplasmic NADH used for glycerol 1-phosphate synthesis
Normal	Insulin	7.24	16.9	7.2
	Insulin + acetate	9.90	12.7	6.5
Starved for 72 h	Insulin	3.68	68.5	15.2
	Insulin + acetate	4.03	52.6	13.9
Starved for 72 h and refed high-fat diet for 72 h	Insulin	3.42	68.4	12.9
	Insulin + acetate	2.48	53.2	16.5
Starved for 72 h and refed bread for 144 h	Insulin	13.70	17.7	5.9
	Insulin + acetate	16.80	20.4	5.2

with adrenaline (Orth, Odell & Williams, 1960; E. D. Saggerson & A. L. Greenbaum, unpublished work). It is thus unclear whether a decrease in pyruvate utilization, or some step subsequent to that, might be the primary site of the lesion in fatty acid synthesis considered in the present work.

In the cytoplasm, a decrease in fatty acid synthesis, whether primarily dependent on diminished pyruvate utilization or on some other mechanism, must result in a decreased utilization of NADPH and hence of NADH transhydrogenation with the result that glycolytic NADH production will be decreased to match its utilization. This therefore introduces a control point into the glycolytic sequence besides the possible citrate control of phosphofructokinase discussed above. This is illustrated by the fact that glucose-carbon flux from triose phosphates to pyruvate is decreased in tissues from starved and starved, fat-refed rats and elevated in those from starved, bread-refed animals (Table 9). It is also noteworthy that, in normal tissues and in those from starved, bread-refed rats, reductive synthesis of lactate and glycerol 1-phosphate account for quite a small proportion (<30%) of the utilization of cytoplasmic NADH (Table 12), whereas in tissues from starved and starved, fat-refed rats these two processes become responsible for the utilization of most of the cytoplasmic NADH (>80%) when fatty acid synthesis rates, and therefore NADH transhydrogenation rates, are low. In this respect glycolysis in adipose tissues from starved and starved, fat-refed rats *in vitro* is similar to that encountered in anaerobic muscle or yeast in that in these systems the process is to a large extent maintained through the reductive synthesis of lactate and ethanol respectively.

In vivo, the linking of triose phosphate oxidation to fatty acid synthesis may be of considerable importance. For example, on ingestion of fat by the parent organism, pyruvate utilization for fatty acid synthesis by adipose tissues may be decreased as discussed above. However, the content of AMP in these tissues may be increased under these conditions as a result of esterification of incoming fatty acids (Saggerson & Greenbaum, 1970), leading to an activation of phosphofructokinase. A second point of control at the level of triose phosphate oxidation would ensure that metabolism of glucose through an activated phosphofructokinase stage was diverted into glycerol 1-phosphate production and that the activation of phosphofructokinase did not lead to a squandering of carbohydrate as lactate and pyruvate which would mainly be released by the tissue. In this context it may be noted that, unlike other fat pad glycolytic enzymes, not only does the triosephosphate dehydrogenase not recover its activity on refeeding a high-fat diet after starvation, but also the activity of this enzyme is decreased on

refeeding the same fat diet without prior starvation (Saggerson & Greenbaum, 1969).

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